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Substrate Independent ATPase Activity May Complicate High Throughput Screening

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Abstract

Inorganic phosphate release, [Pi], is often measured in an enzymatic reaction in a high throughput setting. Based on the published mechanism, we designed a protocol for our screening for inhibitors of SAICAR synthetase (PurC), and we found a gradual increase in [Pi] in positive control samples over the course of the day. Further investigation indicated that hydrolysis of ATP catalyzed by PurC, rather than substrate-related phosphate release, was responsible for a partial contribution to the signals in the control samples. Thus substrate-independent ATPase activity may complicate high throughput screening.

Keywords

Malachite Green; high throughput screening; substrate-independent ATPase activity

SAICAR synthetase (PurC) is involved in *de novo* purine biosynthesis, and is present in most forms of life. As an individual enzyme in many bacterial species, PurC converts L-Asp, 4-carboxy-5-aminoimidazole ribonucleotide (CAIR) and ATP to 4-(N-succino)-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR), ADP and Pi [1–3]. In human, a bi-functional enzyme, PAICS, harbors a PurC domain [4]. PurC has been suggested as a viable target for antimicrobials [5] as well as for chemo-therapeutics [6]. We implemented a Malachite Green assay (MGA) in a high throughput screening (HTS) setup to identify compounds that inhibit the activity of *Bacillus anthracis* PurC (*Ba*PurC). MGA has long been used to monitor the activities of enzymes that release inorganic phosphate by detecting the phosphomolybdate-Malachite Green complex [7,8], and is a common method for the primary screening of inhibitors against enzymes with NTPase activity [9–11].

Our assay was based on the published mechanism, suggesting direct activation of the carboxylate of CAIR by the γ-phosphate of ATP, leading to the formation of products, SAICAR, ADP and Pi [2,15]. After taking into consideration the K_m and K_{cat} values [15], the assay was optimized for HTS and performed in clear flat bottom 384 well plates in 50

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mM Tris buffer at pH 8.0 with 100 mM NaCl, 1 mM $MgCl_2$, 0.01% Triton, and 0.1 mg/mL BSA (Assay Buffer). Each sample well contained purified *Ba*PurC [12] (0.5 μM), L-Asp (2.4 mM), ATP (40 μM) and a specific compound (29 μM) from a small-molecule library (ChemBridge). The compounds were in DMSO, and the DMSO final concentration in the sample was 0.3%. About 40 plates were prepared and screened per day. For each plate, just prior signal detection, the reactions were initiated with the addition of the critical substrate CAIR (20 μM) and allowed to react for 6 min at 25 °C, followed by the addition and 10 min incubati on of Malachite Green dye. The Malachite Green solution was either prepared according to published methods [13,14], or purchased (BioAssay Systems; POMG-25H). The absorbance values at 633 nm of samples in each well were read with a plate reader (Tecan Infinite 200 Pro microplate reader; University of Illinois at Chicago RRC HTS facility), and converted to [Pi] with a calibration curve. We also included positive controls in 32 wells that contained no compound, and negative controls in 32 wells that contained no *Ba*PurC. The overall Z'-factor of the completed runs was 0.85, validating the quality of the assay [16].

In our data analysis, an unexpected trend was noticed. The signals of the positive controls (*Ba*PurC and substrates) did not stay constant during the 8 hr time period each day; instead they steadily increased (Fig 1). The signals of the negative controls (no *Ba*PurC) stayed relatively constant. The reaction scheme is shown in Fig 2 (inset). The results suggested that the signals (Pi concentrations) were not from non-enzymatic ATP hydrolysis, but from a slow enzyme-catalyzed ATP hydrolysis. Intrinsic ATPase activity has been well characterized in protein kinases and is not a new phenomenon [17–20], but would be the first study of this activity in a PurC enzyme.

To further investigate this possibility, samples and controls mimicking HTS conditions were prepared. **Set A** samples in Assay Buffer consisted of *Ba*PurC (0.5 μM), ATP (40 μM) and L-Asp (2.4 mM). After the samples were incubated to 1, 4 or 8 h at 25 °C, CAIR (20 μM) was added and incubated for 6 min, followed by Malachite Green addition and incubation for 10 min. **Set B** samples were similar to **Set A** samples except that CAIR was replaced with buffer, *i.e.* no CAIR in samples. **Set C** samples consisted of CAIR (20 μM), ATP (40 μM) and L-Asp (2.4 mM). After the samples were incubated to 1, 4 or 8 h at 25 °C, *Ba*PurC (0.5 μM) was added and incubated for 6 min, followed by Malachite Green addition and incubation for 10 min. **Set D** samples were similar to **Set C** samples except *Ba*PurC was replaced with buffer, *i.e.* no *Ba*PurC in samples. The absorbance values at 630 nm were obtained with a plate-reader (Perkin-Elmer Victor^3 V 1420 multilabel counter), and converted to the Pi concentrations with a calibration curve.

Results from samples in **Set A** showed an increase in [Pi] as a function of time, from ~7 μM at t = 1 hr, to ~19 μM at t = 8 hr (Fig 2), similar to those observed in HTS. In the absence of CAIR (samples in **Set B**), [Pi] increased from ~3 μM at t = 1 hr, to ~15 μM at t = 8 hr. When *Ba*PurC was added just shortly before measurement (samples **Set C**), a relatively constant [Pi], at ~7 μM, was observed. For samples without the enzyme (**Set D**), a relatively constant [Pi], at ~ 3 μM, was observed. Since 40 μM ATP was present in the reaction samples, we expect that the signals in samples in **Sets A** and **B** will continue to increase beyond 8 h, until all ATP is converted.

Our findings also support our recent proposed mechanism showing that PurC indeed catalyzes ATP hydrolysis to allow a phosphate relay between ATP and conserved Glu residues to activate CAIR [3].

In HTS measuring enzyme activities that involve multiple substrates and co-factors, the decisions on when to introduce which substrates and co-factors to the reaction mixture may be complicated. A logical approach is to design protocols based on published mechanisms. However, as shown in this study, these protocols still need to be carefully tested to provide data that can be meaningfully analyzed. As indicated in recent publications, HTS data sets often have inherent systematic and random error, which may lead to false-positive or false-negative results, and the diversity of the features of different enzymes prevents unification of assay conditions [21–23]. Among many protocols, the assay mixture and blank preparation and the choice of the assay time are crucial to avoid frequent and trivial but costly errors [23].

In this report, we point out potential problems in detecting [Pi] in systems where ATP was a co-factor and not a substrate. Control samples should include samples detecting potential enzyme induced ATP hydrolysis. To our knowledge this is the first detailed analysis showing the pitfall of substrate-independent ATPase activity in PurC systems, and we caution others to take similar enzymatic event into consideration in future experimentation.

Abbreviations

CAIR	4-carboxy-5-aminoimidazole ribonucleotide
HTS	high throughput screening
MGA	malachite green assay
PurC	succinoaminoimidazolecarboxamide ribonucleotide synthethase
SAICAR	succinoaminoimidazolecarboxamide ribonucleotide

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References

1. Levdikov VM, Barynin VV, Grebenko AI, Melik-Adamyan WR, Lamzin VS, Wilson KS. The structure of SAICAR synthase: an enzyme in the de novo pathway of purine nucleotide biosynthesis. Structure. 1998; 6:363–376. [PubMed: 9551557]
2. Ginder ND, Binkowski DJ, Fromm HJ, Honzatko RB. Nucleotide complexes of Escherichia coli phosphoribosylaminoimidazole succinocarboxamide synthetase. J. Biol. Chem. 2006; 281:20680–20688. [PubMed: 16687397]
3. Wolf NM, Abad-Zapatero C, Johnson ME, Fung LW-M. Structures of SAICAR synthetase (PurC) from Streptococcus pneumoniae with ADP, Mg^{2+}, AIR and Asp. Acta Cryst. 2014; D70:841–850.

4. Li S-X, Tong Y-P, Xie X-C, Wang Q-H, Zhou H-N, Zhang Z-Y, Gao W, Li S-G, Zhang XC, Bi R-C. Octameric structure of the human bifunctional enzyme PAICS in purine biosynthesis. J. Mole. Bio. 2007; 366:1603–1614.
5. Yuan Z, Wang L, Sun S, Wu T, Qian W. Genetic and proteomic analysis of a Xanthomonas campestris pv. campestris purC mutant deficient in purine biosynthesis and virulence. J. Genet. Genom. 2013; 40:473–487.
6. Keller KE, Tan IS, Lee Y-S. SAICAR stimulates pyruvate kinase isoform M2 and promotes cancer cell survival in glucose-limited conditions. Science. 2012; 338:1069–1072. [PubMed: 23086999]
7. Itaya K, Ui M. A new micromethod for the colorimetric determination of inorganic phosphate. Clinica Chimica Acta. 1966; 14:361–366.
8. Kiani, FA.; Fisher, S. Molecular catalysts: structure and functional design. Gade, LH.; Hofmann, P., editors. Weinheim, Germany: Wiley-Vch; 2014. Chap.17
9. Bharat A, Blanchard JE, Brown ED. A high-throughput screen of the GTPase activity of Escherichia coli EngA to find an inhibitor of bacterial ribosome biogenesis. J. Biomol. Screen. 2013; 18:830–836. [PubMed: 23606650]
10. Chang L, Bertelsen EB, Wisén S, Larsen EM, Zuiderweg ERP, Gestwicki JE. High-throughput screen for small molecules that modulate the ATPase activity of molecular chaperone DnaK. Anal. Biochem. 2008; 372:167–176. [PubMed: 17904512]
11. Rowlands MG, Newbatt YM, Prodromou C, Pearl LH, Workman P, Aherne W. High-throughput screening assay for inhibitors of heat-shock protein 90 ATPase activity. Anal. Biochem. 2004; 327:176–183. [PubMed: 15051534]
12. Tuntland ML, Wolf NM, Fung LW-M. Differences in the purification and solution properties of PurC gene products from Streptococcus pneumoniae and Bacillus anthracis. Protein Expr. Purif. 2015; 114:143–148. [PubMed: 26118696]
13. Pegan SD, Tian Y, Sershon V, Mesecar AD. A universal, fully automated high throughput screening assay for pyrophosphate and phosphate release from enzymatic reactions. Comb. Chem. & High Throughput Screen. 2010; 13:27–38.
14. Baykov AA, Evtushenko OA, Avaeva SM. A malachite green procedure for orthophosphate determination and its use in alkaline phosphatase-based enzyme immunoassay. Anal. Biochem. 1988; 171:266–270. [PubMed: 3044186]
15. Nelson SW, Binkowski DJ, Honzatko RB, Fromm HJ. Mechanism of action of Escherichia coli phosphoribosylaminoimidazolesuccinocarboxamide synthetase. Biochem. 2005; 44:766–774.
16. Zhang J-H, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 1999; 4:67–73. [PubMed: 10838414]
17. Kaji A, Colowick SP. Adenosine triphosphatase activity of yeast hexokinase and its relation to the mechanism of the hexokinase reaction. J. Biol. Chem. 1965; 240:4454–4462. [PubMed: 4221203]
18. Paudel HK, Carlson GM. The ATPase activity of phosphorylase kinase is regulated in parallel with its protein kinase activity. J. Biol. Chem. 1991; 266:16524–16529. [PubMed: 1832156]
19. Hurley JH. The sugar kinase/heat shock protein 70/actin superfamily: implications of conserved structure for mechanism. Annu. Rev. Biophys. Biomol. Struct. 1996:137–162. [PubMed: 8800467]
20. Kashem MA, Nelson RM, Yingling JD, Pullen SS, Prokopowicz AS III, Jones JW, Wolak JP, Rogers GR, Morelock MM, Snow RJ, Homon CA, Jakes S. Three mechanistically distinct kinase assays compared: measurement of intrinsic ATPase activity identified the most comprehensive set of ITK inhibitors. J. Biomol. Screen. 2007; 12:70–83. [PubMed: 17166826]
21. Mangat CS, Bharat A, Gehrke SS, Brown ED. Rank ordering plate data facilitates data visualization and normalization in high-throughput screening. J. Biomol. Screen. 2014; 19:1314–1320. [PubMed: 24828052]
22. Acker MG, Auld DS. Considerations for the design and reporting of enzyme assays in high-throughput screening applications. Persp. in Sci. 2014; 1:56–73.
23. Bisswanger H. Enzyme assays. Persp. in Sci. 2014; 1:41–55.

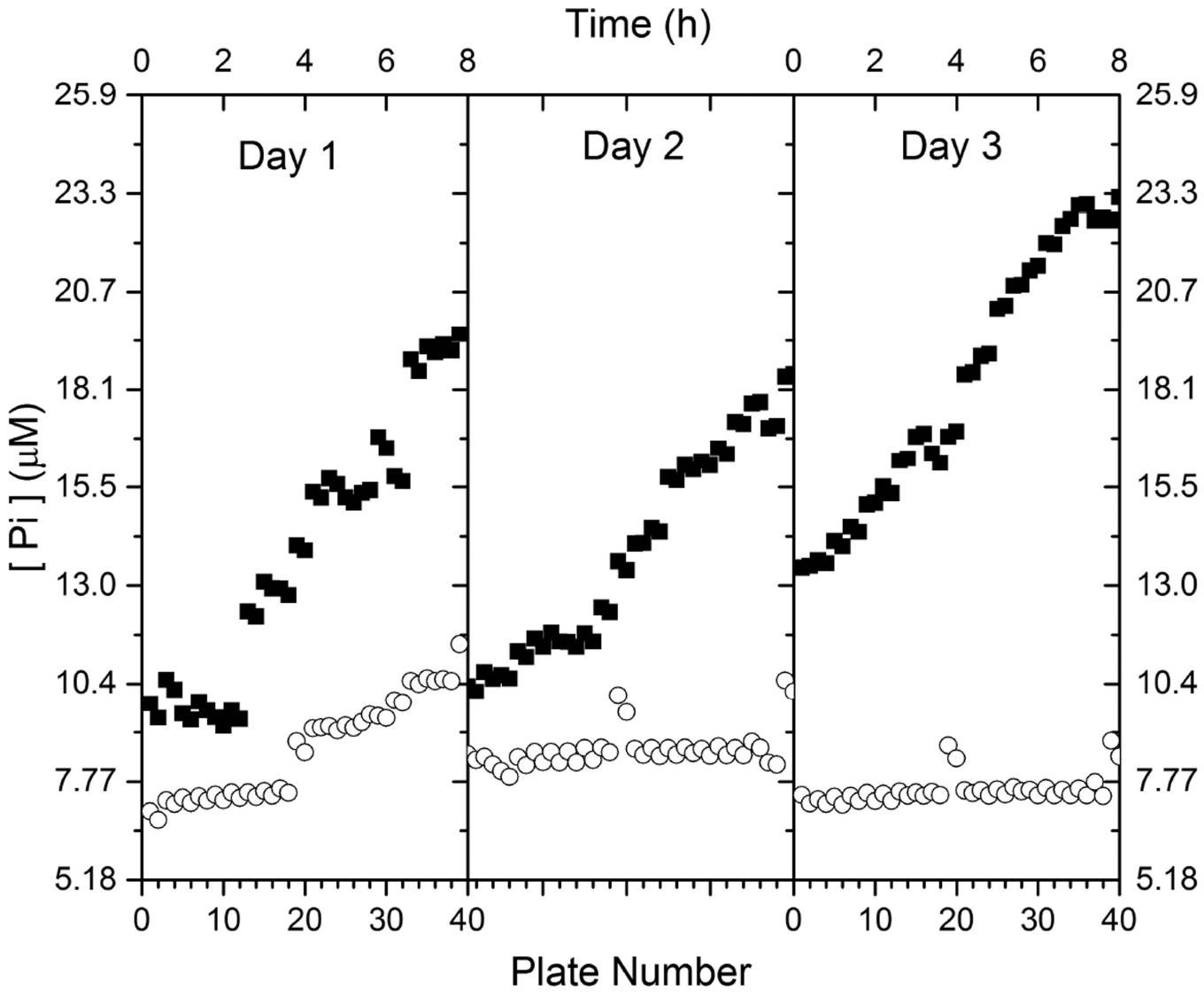

Figure 1. [Pi] of positive controls of *Ba*PurC MGA HTS show a general increase as a function of time

The [Pi] values, from Malachite Green absorbance values, of control samples (n = 32) in each well of the HTS plate, detecting the enzymatic activity of *Ba*PurC. A total of 40 plates were run in a time period of 8 h. Each plate consisted of 32 wells for control samples. The positive control samples (■) consisted of *Ba*PurC (0.5 μM), ATP (40 μM) and L-Asp (2.4 mM) in 50 mM Tris buffer at pH 8.0 with 100 mM NaCl, 1 mM $MgCl_2$, 0.01% Triton, 0.3% DMSO and 0.1 mg/mL BSA. Shortly before each plate was ready for signal detection, CAIR (20 μM) was added and incubated for 6 min. Malachite Green solution was then added and incubated for 10 min. The data for 3 different days are shown. All data of the positive control samples show a gradual rise in the 8 h time period for each of the 3 days. However, the negative control samples (○), which did not include *Ba*PurC, exhibited little or no increase in the [Pi] values.

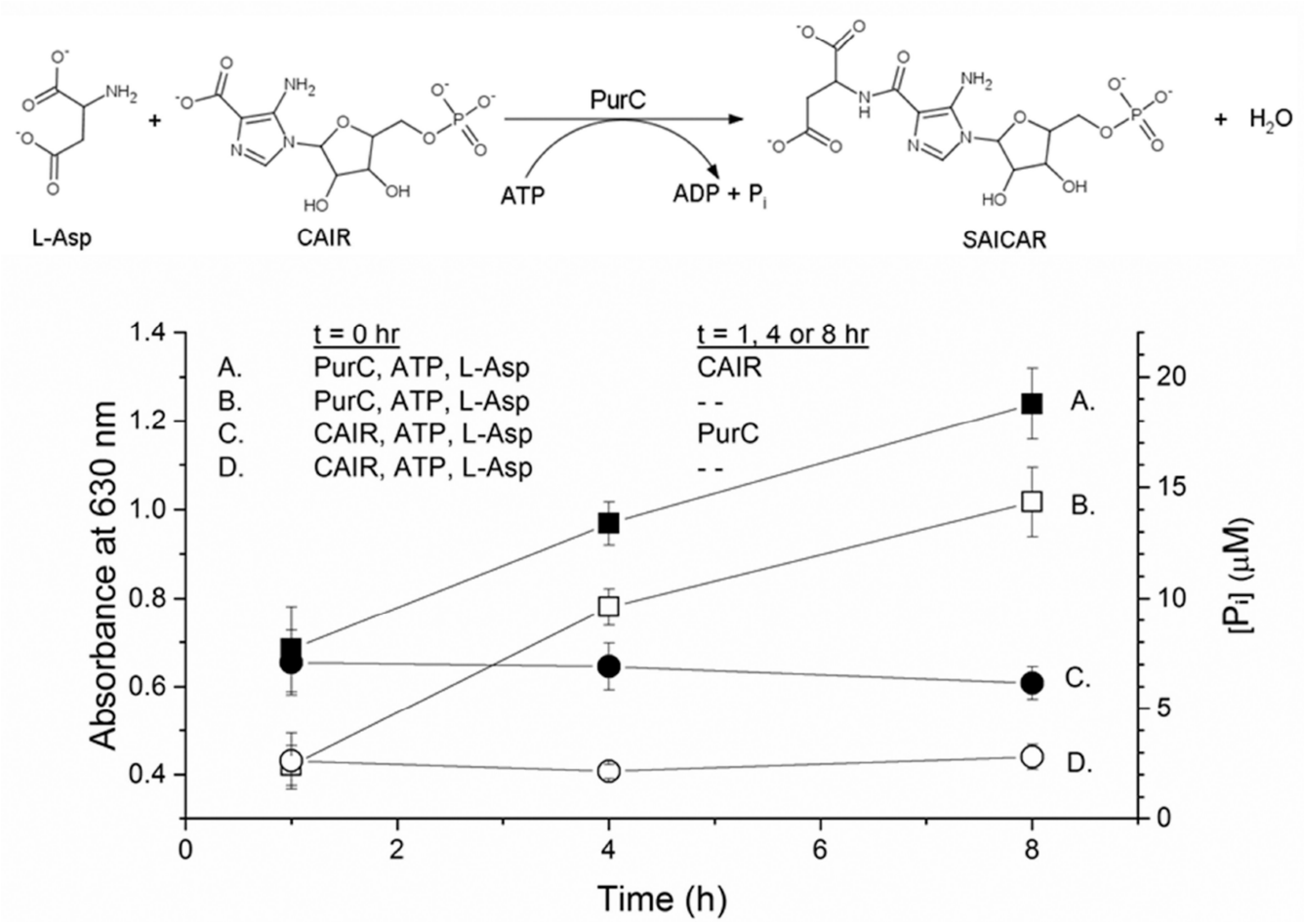

Figure 2. Incubation time and order of assay component addition reveal substrate independent ATP hydrolysis in *Ba*PurC

Tris buffer (50 mM) at pH 8.0 with 100 mM NaCl and 1 mM $MgCl_2$ was used as Assay Buffer for all samples. All three samples in **Set A** (■) consisted of *Ba*PurC (0.5 μM), ATP (40 μM) and L-Asp (2.4 mM). After a sample was incubated to 1, 4 or 8 h at 25 °C, CAIR (20 μM) was added and incubated for 6 min, followed by Malachite Green addition and incubation for 10 min. Samples in **Set B** (□), CAIR was replaced with buffer, *i.e.* no CAIR in the samples. Samples in **Set C** (●) consisted of CAIR (20 μM), ATP (40 μM) and L-Asp (2.4 mM). After a sample was incubated to 1, 4 or 8 h at 25 °C, *Ba*PurC (0.5 μM) was added and incubated for 6 min, followed by Malachite Green addition and incubation for 10 min. For samples in **Set D** (○), *Ba*PurC was replaced with buffer (*i.e.* no *Ba*PurC in samples; similar to the negative control in HTS). The Pi concentration in each sample was obtained from a calibration curve using the absorbance values at 630 nm. For all samples, the data were the averaged values of n = 5 – 10.